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(54) Fibronectins.

(67) The polypeptide sequence of the collagen-binding and fibrin-binding parts of fibronectin and the corresponding cDNA sequences have been determined.

The collagen-binding polypeptide is useful in methods of purification and the fibrin-binding polypeptide is useful for targeting therapeutic substances on natural fibrin.

FIBRONECTINS

Fibronectins (FNs) constitute a class of high molecular weight glyco-proteins that have a key role in various contact processes of the vertebrates such as cell attachment and spreading, cell migration, control of cell 5 morphology, differentiation and oncogenic transformation. All these biological activities imply interaction of FN with cells and with extracellular materials. Binding activities for collagen, heparin, fibrin, cell surfaces, bacteria and DNA have been located in different domains of 10 the FN molecule (for review, see Yamada, 1983).

FN is one of the most versatile known proteins, both functionally and structurally. FN molecules are usually dimers of similar but not identical polypeptides of MW ≈ 250,000. Cellular FN is found in a fibrillar component of 15 the extracellular matrix of fibroblasts and other cell types. Plasma FN is a soluble molecule present in high concentrations in plasma (300 $\mu$ g/ml) and probably involved in opsonization, wound healing and haemostasis (Yamada, 1983; Hynes & Yamada, 1982). Partial primary structure 20 data have revealed high conserved amino acid sequences both between the two FN forms and among FNs from different species: bovine plasma (Petersen *et al.*, 1983), bovine cellular (Kornblihtt *et al.*, 1983), human plasma (Pande & Shively 1982; Garcia-Pardo *et al.*, 1983), human cellular 25 (Kornblihtt *et al.*, 1983, 1984a; Oldberg *et al.*, 1983), rat plasma (Schwarzbauer *et al.*, 1983). These data have tended to confirm that the basic FN polypeptide contains three different types of internal repeats (homology types I, II and III, approximately 40, 60 and 90 amino acids long 30 respectively, as originally shown in bovine plasma FN (Skorstengaard *et al.*, 1982; Petersen *et al.*, 1983). Variations on this basic fibronectin structure account for the differences between cellular and plasma fibronectins and also between the polypeptide chains of both forms.

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The diverse forms of fibronectin seem to be generated by transcription of a single gene into a common precursor which undergoes alternative splicing (Vibe-Pedersen *et al.*, 1984). To-date, at least two regions have been described where this type of variation occurs. In certain human cell lines (fibroblasts, Hs578T) FN mRNAs can be distinguished by a 270 nucleotide segment (ED) that encodes exactly one of the homology type III. This ED segment seems to be absent in the liver hepatocyte mRNAs which are the source of plasma FN (Kornblith *et al.*, 1983, 1984b). Schwarzbauer *et al.* (1983) have reported three different FN mRNAs arising by alternative splicing in rat liver which differ in an area (IIICS) located to the 3' carboxy terminus side in the protein of the ED region. The difference sequence does not belong to any of the known internal homologies and it is inserted between the last two type III homology repeats, near the COOH terminus. In addition, Umezawa *et al.* (1985) have reported further variations in the equivalent IIICS area of human liver FN mRNA, bringing the total to 5 alternative motifs for this area. The differences observed between FN polypeptides are thus the consequence of internal primary sequence variability (Kornblith *et al.*, 1984a 1984b; Schwarzbauer *et al.*, 1983), due to alternative splicing in at least two distinctive regions of the pre-mRNA (Tamkun *et al.*, 1984; Vibe-Pedersen *et al.*, 1984; Umezawa *et al.*, 1985).

The complete amino acid sequence of mature human FN polypeptides has now been determined from the nucleotide sequence of multiple cDNA clones as described below. The polypeptide length varies from 2146 to 2325 amino acids, depending on which internal alternative splicing has taken place.

The present invention thus makes it possible to provide any desired part of the fibronectin molecule and in particular polypeptides having each of the separate binding activities of fibronectin separate from the others.

In the accompanying Figure 2, the binding sites of each part of the FN amino-acid sequence are given. While some of these were previously known, the sequence for the human collagen-binding site (lines 8-13 in the Figure) is new. Lines 9 and 5 10, involving the type II homology, are believed to be of particular significance, and to incorporate most or all of the collagen-binding ability.

The present invention thus provides novel polypeptides having substantially the amino acid sequence from 277 to 577 10 shown in Figure 2, or any continuous part thereof having collagen-binding activity, and especially the sequence from 379 to 445. In practice such polypeptides may be linked to further amino-acid residues not affecting the desired end 15 use of the collagen-binding polypeptide, including additional residues of the fibronectin molecule itself.

Similarly other sequences of the fibronectin molecule may be utilized for their ability to bind to other molecules. Thus, as shown in Figure 2, the polypeptide sequence from 21 to 241 is associated with binding to fibrin, heparin and 20 Staphylococcus aureus. Other sequences are, as shown, associated with binding to DNA, cells and alternative heparin and fibrin binding sites.

The novel polypeptides may be made by culturing cells containing endogenous DNA coding for the polypeptide and 25 separating the polypeptide from the metabolic products. Thus, the appropriate DNA sequences may be cloned into a competent strain of E.coli or other microorganism, e.g. a yeast such as Saccharomyces cerevisiae, the latter cultivated, and then the desired polypeptide isolated from 30 the cultivation products. Figure 3 shows the complete DNA sequence for fibronectin and the associated amino-acid residues, and from this the cDNA sequence required for cloning the expression of any desired part of the fibronectin molecule may be easily determined.

35 The DNA sequence coding for the collagen-binding polypeptide runs from coordinate 1147 to coordinate 1351, and the sequence coding for the fibrin-binding polypeptide runs from coordinate 73 to 738.

More particularly, however, in accordance with the present invention,

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the clones herein described as pFH54, pFH134, pFH16 and pFH6 and similar clones may be used to produce corresponding polypeptides by expression in E. coli or other appropriate microorganisms. pFH134 and pFH16 contain the DNA sequence for 5 the collagen-binding part of the fibronectin molecule and may be used to generate a polypeptide having the collagen-binding activity of fibronectin without its other binding affinities. pFH6 can be used to transform competent E. coli for the expression of a polypeptide binding fibrin and 10 heparin.

The manner in which the clones pFH54, pFH134, pFH16 and pFH6 were obtained is described below, and repetition of the methods referred to will give similar clones with essentially the same or only slightly different 15 utility. It will be appreciated in this connection that the isolation of useful cDNA sequences using the total cellular RNA from an appropriate source, i.e. cells naturally capable of expressing the desired protein or polypeptide, is a matter of routine experimentation for 20 the person skilled in the art using the currently available techniques, particularly when, as is the case here, the actual amino acid sequence for the desired protein or polypeptide and the corresponding DNA sequence are both known. The experimental section below mentions appropriate 25 techniques which have been found to be effective but it is to be expected that other known techniques would be equally applicable. Similarly choice of appropriate sources of RNA, vectors, and competent microorganisms for transformation from the many materials which are now 30 available to the experimentalist is well within the ordinary skill in this art.

It will be appreciated that when a desired amino-acid sequence of fibronectin is expressed by a transformed microorganism it may be associated with a polypeptide 35 characteristic of the microorganism itself. This may be immaterial to the intended use of the polypeptide but, in some cases, eg. if the polypeptide is to be used in therapy,

the presence of the additional amino-acid residues may be unacceptable. In that case the polypeptide must be subjected to an additional treatment, e.g. with a protease, to separate the desired polypeptide free from undesired 5 additional amino-acid residues.

As indicated above, the present invention is of especial interest in that it provides a means for transforming an appropriate microorganism to make it capable of expressing a polypeptide able to bind to collagen and/or 10 fibrin. A polypeptide able to bind to collagen can be used, for example, to facilitate affinity purification of valuable polypeptides. Thus if the collagen-binding polypeptide is expressed in a form in which it is bound to another polypeptide of interest or if it is linked, after 15 isolation, to such a polypeptide, the combined polypeptides may be purified by affinity chromatography on a column of bound gelatin (i.e. collagen), and then, after the purification, the desired polypeptide may be separated, e.g. by an enzymatic hydrolysis, from the collagen-binding 20 polypeptide.

A polypeptide able to bind to fibrin may be used in therapy to target a therapeutic agent on natural fibrin, e.g. a blood clot. For example, a fibrinolytic enzyme bound to the polypeptide would have improved clot-dissolving properties, since it would have improved adhesion to its target. 25

In the accompanying drawings, Fig. 1 shows a restriction enzyme map of seven cDNA clones covering 76% nucleotides from the poly(A) tail of human FN mRNA. 30 Human FN mRNA has been estimated to be 7900 nucleotides long (Kornblith *et al.*, 1983). The clones cover the complete coding region for the mature protein (bottom diagram showing binding sites) and the 3' non-coding region. The dotted lines indicate segments that are absent from the corresponding cDNA clones but that must have been

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synthesized in the first strand cDNA reaction and that were lost as a result of the failure of the Klenow enzyme to complete the second cDNA strand. Numbering on the map is in base pairs.

5        Fig. 2 shows the complete amino acid sequence of a human FN polypeptide. Residues 1 and 2325 are the NH<sub>2</sub> and COOH termini of the mature protein. The sequence was deduced from the nucleotide sequence (see Figure 3) of the cDNA clones depicted in Fig. 1. Alignment shows  
10 internal homologies. Gaps were introduced to maximize the homology. Identical residues within a type of homology are boxed. The cell recognition tetrapeptide RGDS (Pierschbacher & Ruoslahti, 1984) is underlined. Positions 17 (Ser), 21 (Cys) and 42 (Val) are reported as Cys, Ser  
15 and Ala respectively by Garcia-Pardo *et al* (1983). The FN polypeptide shown in this Figure has 2325 residues with a MW = 255,905. If the mass contributed by the carbohydrate side chains, estimated to be 9% of the protein mass (Yamada, 1983), is added, the molecular weight of this  
20 FN polypeptide would increase to approximately 279,000. This figure appears to be considerably higher than the weights of the FN monomers estimated by SDS-PAGE (230-250,000). The discrepancy could be explained by the poor resolution of the SDS gels in the range of high molecular weight proteins  
25 together with the lack of appropriate protein standards in that range.

Symbols are as follows: ■, free SH groups;  $\lambda$ , sites for carbohydrate side chains;  $\Delta$ , cleavage site for chymotrypsin.  $\blacktriangle$ , cleavage site for plasmin. The multiple  
30 fibronectin polypeptides can be generated by all the possible permutations of the alternative splice regions in lines 26 and 30 (as explained below).

Fig. 3 shows the complete nucleotide sequence for the human FN polypeptide of Figure 2 deduced from the  
35 sequences of the cDNA clones of Figure 1.

Fig. 4 shows the variations of the FN primary structure.

... is the complete structure of the mature protein. Black boxes are type I homologies; dashed boxes, type II homologies;

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mpty boxes, type III homologies.

B and C show schematic representation of the different FN polypeptides that could arise by the translation of the multiple FN RNAs observed in the ED region (B) and in the IIICS region (C). The name of the cDNA clones representing the corresponding encoding mRNA species is shown to the right of each polypeptide.  $\wedge$  indicates contiguity.  $\lambda$ rflf2, 4 and 6 were isolated from a rat liver cDNA library (Schwarzauer *et al.*, 1983), pFHLL and 8 from a human liver cDNA library, and pFH1 was isolated from the Hs578T cell line cDNA library (Kornblith *et al.*, 1983). It will be noted that all the variations include the zones binding to collagen and fibrin.

Figure 5 shows, in more detail than Figure 1, part of the fibronectin protein indicating the position of the collagen-binding domain and internal homologies (I, II, and III). Below, the position and sizes (in base pairs) of the series of cDNAs used in the bacterial expression experiments described below is indicated. Only the restriction enzyme sites relevant to the cDNAs pXFN1-8 are shown. The flanking Hind III and Bam HI sites of pFH134 and pFH16 occur in the polylinker of the vector.

Figure 1 shows the restriction map of different cDNA clones covering the 3' non-coding region and the complete coding region for the mature protein of human FN mRNA. The isolation of clones pFH1, pFH11 and pFH154 has been previously described (Kornblith *et al.*, 1983, 1984a) and nucleotide sequence and deduced amino acid sequence of the latter has been published before (Kornblith *et al.*, 1984b). The clones pFH54, pFH134, pFH16 and pFH6 are new. Isolation of these four cDNA clones covering the 5' third of the map involved the synthesis of an oligonucleotide primer. The sequence (namely 5-GCTGAACCATTTGCTGAGC) of the primer was complementary to the mRNA sequence of a region close to the 5' end of clone pFH154. The oligonucleotide was used to prime reverse transcription of total RNA from Hs578T cells (Hackett *et al.*, 1977) and a cDNA library was prepared as described below. The clones pFH54, pFH134, pFH6 and pFH16 were selected for further analysis. The complete nucleotide sequence of these clones was determined and comprised 7692 bp, of which

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6972 bp correspond to the coding region and 720 bp to the 3' nontranslatable region and poly(A)tail. The sequence is included in the full DNA sequence for FN shown in Figure 3.

The amino acid sequence of human fibronectin deduced from the nucleotide sequence of the clones in Figure 1 is shown in Figure 2. The alignment in Figure 2 maximises internal homologies. The complete FN chain presents regions which have 3 different types of internal homologies (types I, II and III) (Petersen *et al.*, 1983) and regions which have no homologous counterparts within the molecule. The latter are the NH<sub>2</sub>-terminal and COOH-terminal segments and the internal connecting strands. From NH<sub>2</sub>-to COOH- terminus, FN is formed by one 20-residue long NH<sub>2</sub>-terminal segment (Fig. 2, line 1), five units of type I homology or fingers (lines 2 to 6), one connecting strand (line 7), one finger (line 8), two units of type II homology (lines 9 and 10), three fingers (line 11, 12 and 13), one unit of type III homology (line 14), one connecting strand (line 15), fourteen units of type III homology (lines 16 to 29, including the ED polypeptide), one connecting strand (IIICS, line 30), one unit of type III homology (line 31), one connecting strand (line 32), three fingers (lines 33, 34 and 35) and the COOH- terminal segment (line 36).

The primary structure of FN reflects a level of order and complexity not seen before in any other protein. The symmetry in the array of the 16 units of type III homology is particularly interesting. Two type III units (Fig. 2, lines 14 and 31) are separated by connecting strands (lines 15 and 30) from a central block, having the remaining fourteen in a juxtaposed way. The degree of homology within the type III units is very high. Three residues are conserved in all the units, *viz* Trp (Fig. 2, box with residue 599 at the top), Leu (box with residue 640 at the top) and Tyr (box with residue 646 at the top). The conserved residues are distributed in two peaks around the Trp and the Tyr, separated by a valley of non homology. It is believed that the degree of order and conservation in

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the type III sequences must reflect particular constraints of the secondary structure of the central region of FN. This region is not stabilized by disulphide bridges since the only two Cys residues present in the type III sequences (positions 1201 and 2075 in 5 Figure 2) have been shown to exist in a reduced form. (Vibe-Pedersen *et al.*, 1982; Smith *et al.*, 1982).

Several binding activities have been assigned to different regions of the FN molecule (see Figs 1 and 2). However, only in the case of the ability to bind cells, has 10 the actual binding site been identified so far. In fact, Pierschbacher and Ruoslahti (1984) demonstrated that the tetrapeptide Arg-Gly-Asp-Ser (RGDS) is responsible for the cell attachment activity of FN. This tetrapeptide is present only once in the sequence of Fig. 2 at positions 15 1493 to 1496, within one of the type III units. Fig. 2 also shows that the optimal alignment of the type III sequences in this area is obtained only if the tetrapeptide is considered to be an extra element, allowing four gaps in the corresponding regions of the rest of the type III 20 units. It is probable that, as well as the cell binding site, other binding sites or biological activities within type III sequences reside in non-conserved stretches. The tetrapeptide has also been found in other proteins (Pierschbacher & Ruoslahti, 1984) including the  $\alpha$  chain of 25 fibrinogen which shows cell attachment activity.

An important feature of FN gene expression is the generation of slightly different polypeptides by differential processing of the common mRNA precursor (Vibe-Pedersen *et al.*, 1984). Figure 4A shows diagrammatically 30 the localization of the two regions of variability observed so far along the FN molecule (Schwarzauer *et al.*, 1983; Kornblith *et al.*, 1984a 1984b). Figures 4B and 4C show the types of polypeptide that can arise from the translation of the different mRNAs generated in the ED 35 (Fig. 2 line 26) and IIICS (Fig. 2, line 30) regions respectively. This diagram combines observations made on

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both human and rat fibronectin. At least 10 different FN polypeptides can be generated from a single gene if it be assumed that all the permutations between the ED and IIIICS segments are possible. This is consistent with the 5 FN polypeptide heterogeneity observed in the bidimensional gel electrophoresis analysis of cellular and plasma FNs found in vivo. Homo- or hetero- dimeric FN molecules can then be formed from the FN polypeptide pool. The biological significance of this complex situation is not 10 yet clear. However, it is to be noted that the ED and IIIICS variable regions are intercalated between the cell-heparin and heparin-fibrin binding sites. The distance between these biologically active sites of the molecule may be critical for the FN function. For example, plasma 15 FN is 1 to 2 orders of magnitude less active than cellular fibronectin in restoring morphology and alignment to a transformed fibroblast cell line (Yamada & Kennedy, 1979). Further the mRNAs carrying the ED segment are present in fibroblasts (one source of cellular FN) but not in liver 20 cells (one source of plasma FN) (Kornblith et al, 1984b). It is possible that the function of the ED is to increase the distance between the cell binding tetrapeptide and the heparin binding site, resulting in an enhanced binding activity of the cellular FN molecule.

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### EXPERIMENTAL

#### RNA Preparation

Human cell line Hs578T (Hackett *et al.*, 1977) was cultured in Dulbecco's modified Eagle's medium containing 10% foetal calf serum. Total RNA was extracted from confluent cell monolayers by the guanidine-HCl method 5 (Chirgwin *et al.*, 1979). Between 2 and 4 mg of total RNA were extracted from  $4 \times 10^8$  cells.

Other sources of RNA could be used if preferred, eg. fibroblasts or liver cells.

#### Isolation of fibronectin cDNA clones

All the cDNA clones depicted in Figure 1 were 10 obtained using Hs578T cell RNA as template. Isolation of clone pFH1 by oligonucleotide probing was described by Kornblihtt *et al.* (1983). Isolation of clones pFH111 and pFH154 by "mRNA walking" (oligonucleotide priming) was described by Kornblihtt *et al.* (1984a). This latter 15 procedure was used for the isolation of the new clones pFH54, pFH134, pFH16 and pFH6. An oligonucleotide primer complementary to the mRNA region close to the 5' end of pFH154 was synthesized by the method of Gait *et al.* (1980). The oligonucleotide was used to prime reverse transcription 20 of total RNA from Hs578T cells (Hackett *et al.*, 1977). Blunt ended ds cDNA was prepared and cloned into the plasmid pAT153/PvuII/B (Anson *et al.*, 1984) in *E. coli* MC1061 as previously described (Kornblihtt *et al.*, 1983). Colonies were screened using as probe a restriction 25 fragment from the 5' end of pFH154 lacking the primer sequences, labelled by filling in at one end. In this way, clones pFH54 and pFH134 were obtained. In a second st p, clones pFH16 and 6 were obtained by screening with an end labelled probe for the 5' end of clone pFH134.

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Restriction fragments of the fibronectin cDNA were filled in with the Klenow fragment of DNA polymerase I and blunt end ligated into SmaI cut/phosphatased pEX 1, 2 or 3 vector. Transformations were carried out using the E. coli strain 5 LKIII (Zabeau et al., 1982) harbouring the plasmid pcl857 which specifies kanamycin resistance and carries the cl857 allele (Remaut et al., 1983). Colonies were transferred to Whatman 541 filter paper (Gergen et al., 1985) and screened with either 3' end labelled (Maxam et al., 1977) or nick 10 translated probes (Rigby et al., 1977).

#### Sequence determination

Inserts from clones were excised from the vector DNA by digestion with appropriate restriction enzymes, separated in agarose gel electrophoresis, and recovered by electroelution 15 (Girwitz et al., 1980). Most of the sequencing was performed by the chemical degradation procedure of Maxam and Gilbert (1980). Some regions were sequenced by the chain terminator method (Sanger et al., 1977). For that purpose, the relevant fragments were isolated, digested either with AluI or HaeIII 20 and ligated to a SmaI digested M13mp9 vector (Messing & Vieira, 1982), previously treated with calf intestinal phosphatase to prevent its circularization. The ligation mixtures were used to transform competent E. coli JM101 and recombinants were selected as clear plaques by insertional 25 inactivation of the  $\beta$ -galactosidase gene (Messing et al., 1981). Single stranded DNA was prepared by standard procedures (Winter & Fields, 1980) and the inserts were sequenced using a "universal" 17-nucleotide long primer (Duckworth et al., 1981).

#### Preparation of bacterial extracts

30 Bacteria carrying recombinant plasmids were grown at 30°C for 2½h and expression of the cro/ $\beta$ -galactosidase fusion protein induced by shifting to 42°C for 2h. Bacteria were pelleted at 1200 g and washed with 50 mM TrisHCl, pH 7.4, 170 mM NaCl. Cells were resuspended in the same buffer 35 containing lysozyme (2.5 mg/ml) and sonicated for 2 min on ice. The lysate was centrifuged at 45,000 g for 30 min at

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4°C. The pellet was resuspended in 7 M urea in 10 mM Tris HCl, pH 7.4, 1mM EDTA and incubated at room temperature for 30 min. The solubilised extract was dialysed extensively against 50 mM Tris HCl pH 7.4 at 4°C, and then centrifuged 5 at 45,000 g for 30 min at 4°C.

#### Gelatin-Sepharose chromatography

Gelatin-Sepharose was either obtained from Sigma Chemicals (St. Louis, MO, USA) or prepared by linking gelatin (pig skin type I, Sigma Chemicals) to CNBr-activated 10 Sepharose CL.4B (Pharmacia, Uppsala, Sweden). Chromatography of bacterial extracts on gelatin-Sepharose was carried out as described by Ruoslahti *et al* (1982). The efficacy of the gelatin-Sepharose matrix was verified using purified human plasma fibronectin (Sigma Chemicals).

#### 15 Electrophoretic analysis

SDS-polyacrylamide gel electrophoresis was carried out in 0.1% (w/v) SDS in Tris/glycine buffer on 7.5% (w/v) acrylamide slab gels (19). Gels were stained with 0.1% Coomassie blue in methanol/water/acetic acid (4:5:1 by vol.). 20 Immunoblotting was performed as described by Towbin *et al.* (1979). Polypeptides, electrophoretically transferred to nitrocellulose were probed with rabbit anti(human plasma fibronectin) serum (1:500 in phosphate buffered saline, 10% newborn calf serum and 0.05% Tween 20). Bound immunoglobulin 25 was visualised using alkaline phosphatase conjugated goat anti-(rabbit IgG) (1:1000; Sigma Chemicals).

#### Protein assay

Protein was estimated by the method of Bradford (1976) using bovine serum albumin as a standard.

#### 30 Construction and characterisation of fibronectin expression plasmids

The human fibronectin cDNA clones, pFH134 and pFH16, encompass all or part of the collagen-binding domain of fibronectin identified by proteolytic cleavage of the protein (see Fig. 1 and Fig. 5). These cDNAs were therefore chosen 35 as the starting point for investigating the expression of a functional collagen-binding site in E. coli. The pEX vectors

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used for cloning enable exogenous gene sequences to be inserted into a polylinker in all three reading frames at the 3' end of a cro-LacZ hybrid gene under the control of the  $\lambda P_r$  promoter (Stanley *et al*, 1984). The 5' ends of the 1.74 kb and 1.04 kb inserts of pFH134 and pFH16 respectively were sequenced (Maxam *et al*, 1980) to establish the reading frames of the cDNAs and blunt end cloned into the SmaI site of pEX2. The recombinant plasmids were introduced into an E. coli strain previously transformed 10 with a plasmid encoding the temperature-sensitive  $\lambda P_r$  repressor, c1857. This allows for temperature-inducible expression of the cro/ $\beta$ -galactosidase protein. To test for the production of fibronectin fusion protein by the expression constructs, hybridisation positive clones were 15 grown at 30°C for 2½ h and then shifted to 42°C for a further 2 h. Total bacterial lysates were analysed by SDS polyacrylamide gel electrophoresis. Five of ten pXFH134 constructs and one of seven pXFH16 constructs showed the production of high molecular weight polypeptides of sizes 20 consistent with the lengths of the cDNA inserts (~ 185 kD and ~ 165 kD respectively. The correct orientation of the fibronectin sequences in pXFH134 and pXFH16 was confirmed by restriction enzyme analyses.

The fusion proteins produced by pXFH134 and pXFH16 accounted for approximately 20% of the total bacterial protein consistent with the previous report for this vector system (Stanley *et al*, 1984). Both fusion proteins showed some proteolytic degradation, particularly the pXFH134 polypeptide, which appeared to be partially cleaved to the size of the wild-type cro/ $\beta$ -galactosidase (116 kD). Analysis 30 of proteins synthesised over a time course of induction (0 to 120 min) indicated that proteolysis occurred concomitantly with synthesis of the fusion proteins.

The expression of fibronectin antigenic determinants 35 in pXFH134 and pXFH16 was investigated by immunoblotting using a rabbit polyclonal anti-(human plasma fibronectin) serum.

The antiserum reacted with the 185 kD polypeptide synthesised by pXFH134 but not with the pXFH16 fusion protein or the cro/β-galactosidase polypeptide, indicating that the epitope(s) recognised by the anti-serum lie outside the type II homology units and adjacent type I repeats (Fig. 5). This observation is consistent with the poor antigenicity of the collagen-binding domain of human fibronectin previously reported (Ruoslahti *et al.*, 1979) and most probably reflects the very high level of amino acid conservation in this region (Fig. 2).

Gelatin-Sepharose affinity chromatography

Over-production of β-galactosidase fusions in *E.coli* results in the precipitation of the protein in the cells as insoluble inclusion bodies (Williams *et al.*, 1982, Cheng 1983, Stanley 1983). Thus, when bacteria expressing the pXFH134 plasmid were lysed by sonication and centrifuged, the fibronectin fusion protein was found exclusively in the insoluble pellet. This fraction represented approximately 50% of the total protein of the bacterial lysate. Solubilisation of this material required treatment with 7 M urea and, following dialysis, 60% of the protein remained in solution. This fraction, which was highly-enriched in the fusion protein, was applied directly to a 5 ml gelatin-Sepharose column equilibrated in 50 mM Tris HCl, pH 7.4. The column was washed with 0.5 M NaCl in 50 mM Tris HCl, pH 7.4 until the  $E_{280}$  of the flowthrough was <0.01. The cro/β-galactosidase-fibronectin hybrid protein was eluted from the column as a single symmetrical peak with 4 M urea in the same buffer. Under these conditions fibronectin is specifically released from gelatin-Sepharose (Ruoslahti *et al.*, 1982). In the control experiment using pEX2 only, no binding of the wild-type cro/β-galactosidase protein was observed.

A functional collagen-binding site has therefore been reconstituted in the pXFH134 fusion protein. It must be noted, however, that the fusion protein specifically eluted from the column represented < 5% of the fusion protein

applied to the column. Thus, not surprisingly, considerable activity is lost due to the insolubilisation of the fusion proteins in the bacterial cells, and subsequent vigorous treatment required to resolubilise them.

5       The fibronectin fusion protein produced by pXFH16 was also tested for gelatin-binding and showed similar activity to pXFH134. This indicated that the collagen-binding region occurred within the domain defined at the protein level and strongly implicated the two type II and adjacent type I  
10 homology units (see Fig. 5). To further localise the binding site, a series of overlapping expression constructs was made from pFH16 (Fig. 5) and systematically assayed for gelatin-binding activity. The results are summarised in Table 1, and show the consistent involvement of the type II homology  
15 units (pXFN 2, 3 and 6).

Table 1

pEX construct	Binding to Gelatin-Sepharose
pXFH134	+
pXFH16	+
pXFN1	-
2	+
3	+
4	-
5	-
6	+
7	-
8	-
pEX2 vector only	-

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The binding activity of pXFH134 is almost entirely accounted for by a construct consisting of the two type II homology units (pXFN3). By comparing the gelatin-binding activity of pXFN3 and pXFN6 (both active) with pXFN5 and pXFN8 (both 5 inactive), it may be deduced that the amino acid sequence critical for binding lies in the C-terminal half of the fibronectin fragment in pXFN3, and more particularly from the *HinfI* site (coordinate 1147 of Figure 3) to the *RsaI* site (coordinate 1351 of Figure 3). This 66 amino acid 10 sequence represents almost the entire second type II homology unit of fibronectin plus a few amino acids of the adjacent type I homology unit (see Fig. 2).

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CLAIMS

1. A polypeptide sequence having specific affinity for collagen and/or fibrin and containing amino-acid residues in substantially the same order as in the amino-acid residues in the collagen binding and/or fibrin binding part of fibronectin.
2. A polypeptide according to claim 1 comprising at least the collagen-binding portion of the amino-acid sequence 277 to 577 shown in Figure 2.
3. A polypeptide according to claim 1 comprising at least the collagen-binding amino-acid sequence 379 to 445 shown in Figure 2.
4. A polypeptide according to claim 1 comprising at least the fibrin-binding portion of the amino-acid sequence 21 to 241 shown in Figure 2.
- 15 5. A polypeptide according to any of claims 1 to 4 bound to a polypeptide sequence not present in fibronectin.
6. Method of purifying a substance which comprises contacting a conjugate of that substance and the collagen-binding portion of fibronectin with immobilized collagen 20 so that the said conjugate binds to the said collagen, and then eluting the said conjugate.
7. Method according to claim 6 in which the said eluted conjugate is then split to remove the said collagen-binding portion, and the said substance is then isolated.
- 25 8. A polypeptide according to claim 1 or 4 comprising a fibrin-binding amino-acid sequence bound to a therapeutic agent.
9. A cDNA sequence coding for the polypeptide sequence of fibronectin having specific affinity for collagen and/or 30 fibrin.
10. A cDNA sequence according to claim 9 having the structure shown at 1147 to 1351 in Figure 3b.
11. A cDNA sequence according to claim 9 having the structure shown at 73 to 738 in Figure 3a.
- 15 12. A plasmid or other vector containing a cDNA sequence

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as defined in any of claims 9 to 11.

13. A microorganism modified by inclusion of a vector  
as claimed in claim 12.

14. Escherichia coli modified by inclusion of a  
vector as claimed in claim 12.

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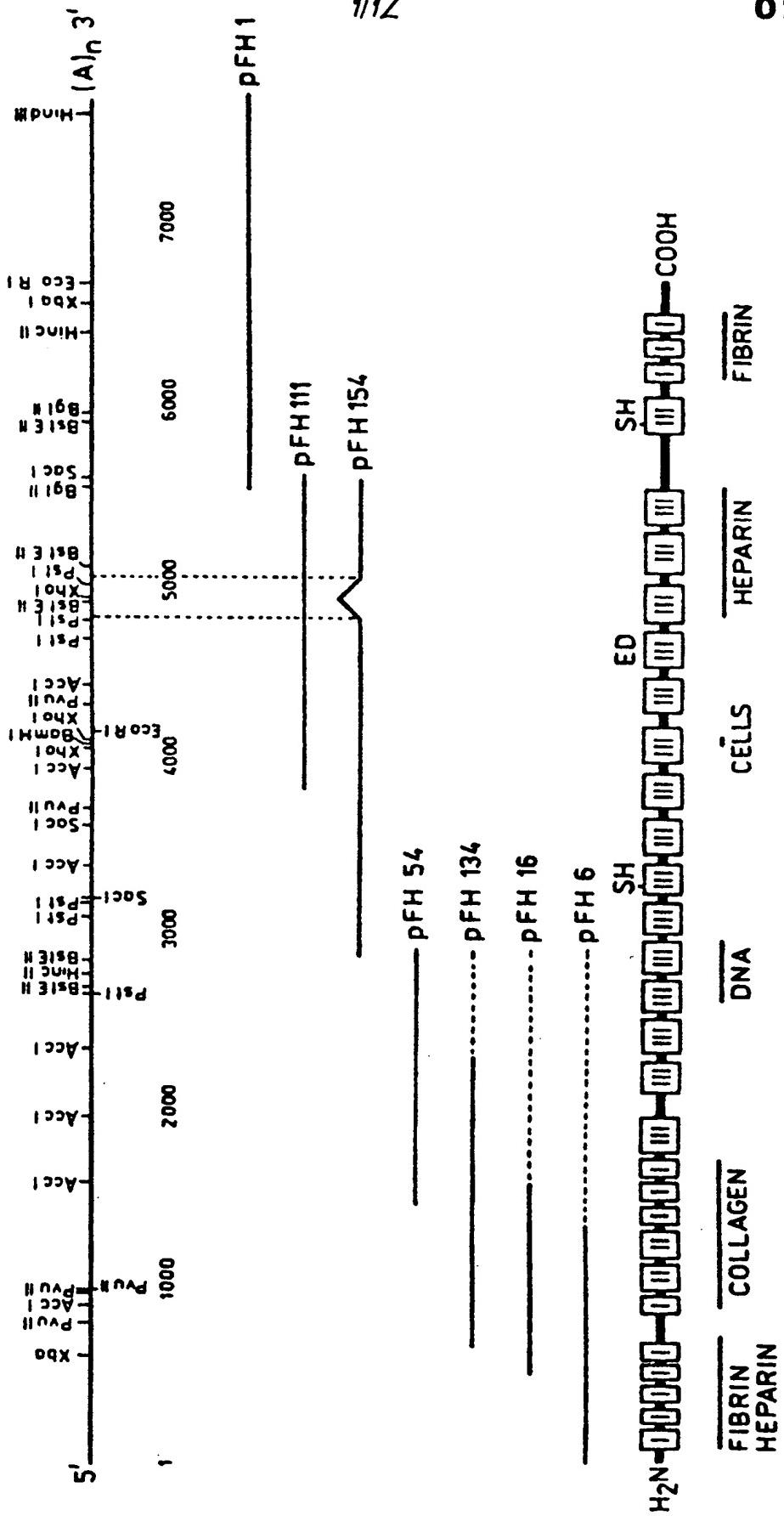
CLAIMS FOR DESIGNATED STATE AT

1. Process for producing a polypeptide sequence having specific affinity for collagen and/or fibrin and containing amino-acid residues in substantially the same order as in the amino-acid residues in the collagen binding and/or fibrin binding part of fibronectin, which comprises culturing a cell containing an exogenous DNA sequence coding for said polypeptide sequence, and separating said polypeptide sequence from the metabolic products of said cell.
- 10 2. A process according to Claim 1 in which the said polypeptide comprises at least the collagen-binding portion of the amino-acid sequence 277 to 577 shown in Figure 2.
3. A process according to Claim 1 in which the said polypeptide sequence comprises at least the collagen-binding amino-acid sequence 379 to 445 shown in Figure 2.
- 15 4. A process according to Claim 1 in which the said polypeptide sequence comprises at least the fibrin-binding portion of the amino-acid sequence 21 to 241 shown in Figure 2.
- 20 5. A process according to Claim 3 in which the exogenous DNA has the structure shown at 1147 to 1351 in Figure 3b.
- 25 6. A process according to Claim 4 in which the exogenous DNA has the structure shown at 73 to 738 in Figure 3a.
7. Method of purifying a substance which comprises contacting a conjugate of that substance and the collagen-binding portion of fibronectin with immobilized collagen so that the said conjugate binds to the said collagen, and 30 then eluting the said conjugate.
8. Method according to Claim 7 in which the said eluted conjugate is then split to remove the said collagen-binding portion, and the said substance is then isolated.

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Fig. 1.



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BINDING SHEET

Fig. 2.

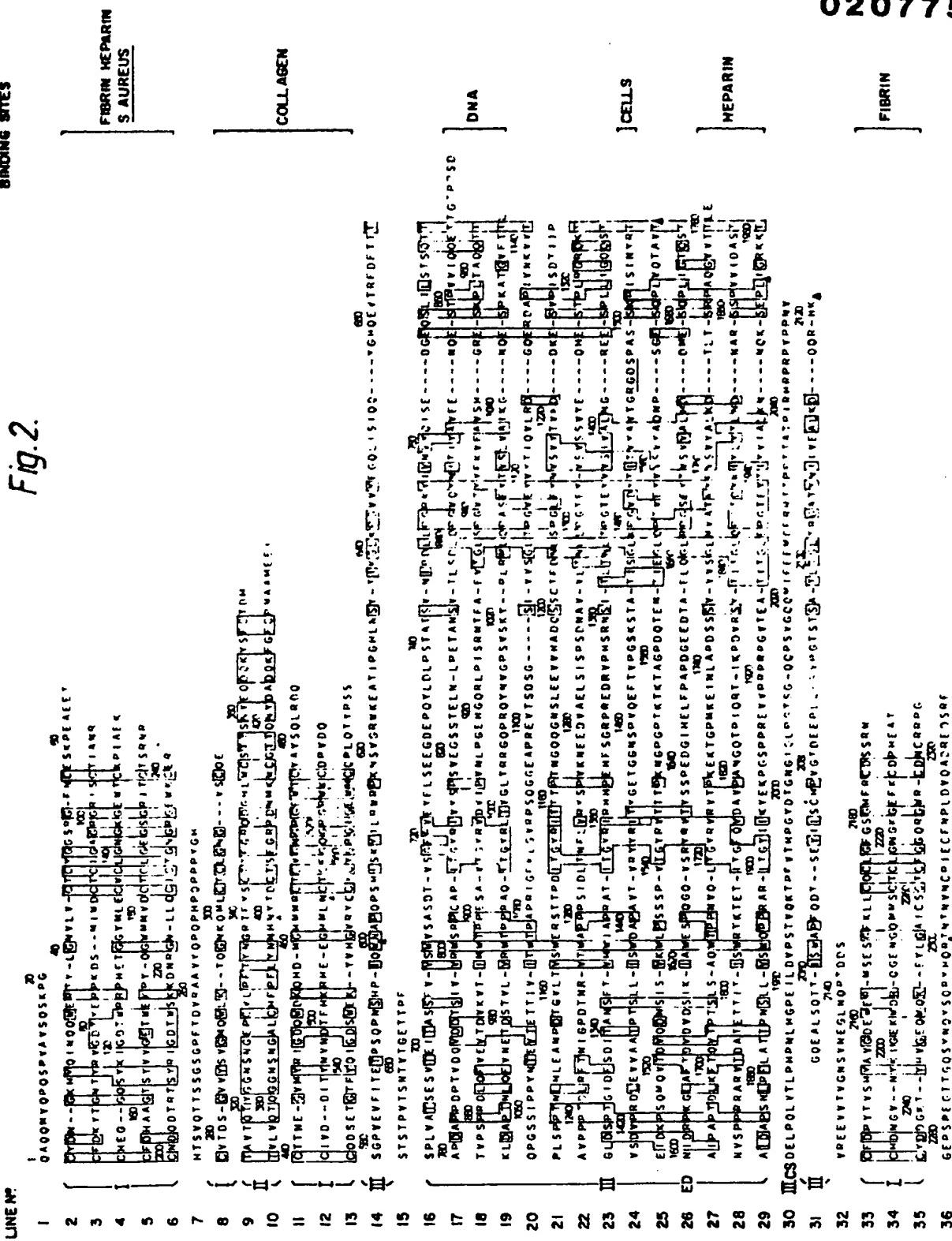


Fig. 3a.

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*Fig. 3b.*

T O T Y G G N L N G E P C V L P F T Y N G R T F Y S C T T E G R Q D G H L W C S  
 AACCCAGACTTACGGTGCACATTAACTGAGGCCATTACCTTACCTTACCAATGGCAGGTTCTACTCTGACCCAGGAGGACATCTTGGTCAG  
 970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080  
 10  
 T T S N Y E Q O Q K Y S F C T D H T V L V Q T Q G G N S N G A L C H F P F L Y N  
 CACACACATTACACTGATTGACCTTCTGAGGGCAGAACAGAACATGAACTGAGGTGTTGGTCAACTGTTGGGAAATTCCAACTGCTGCTCTTCTATACAA  
 1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200  
 N H N Y T D C T S E G R R D N M K W C G T T Q N Y D A D Q K F G F C P M A A H E  
 CACACACATTACACTGATTGACCTTCTGAGGGCAGAACAGAACATGAACTGAGGTGTTGGTCAACTGTTGGGAAATTCCAACTGCTGCTCTTCTATACAA  
 1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320  
 11  
 E I C T T N E G V M Y R I G D O W D K Q H D N G H M M R C T C V G N G R G E W T  
 GGAATCTGCACAACTGAAAGGCTCATGTAACCCATTGAGGATAGGGATAAGCAGCATGACATGGGTGCACTGATGGGAAATGCTGTGGGAAATGGAC  
 1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440  
 C I A Y S Q L R D Q C I V D D I T Y N V N D T F H K R H E E G H M L N C T C F G  
 ATCCATTGGCCTACTGACATGAGGTTGACATTGAGGTTGACATTGAGGTTGACATTGAGGTTGACATTGAGGTTGACATTGAGGTTGACATTGAGG  
 1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560  
 12  
 Q G R G R W K C D P V D Q C Q O S S E T G T F Y Q I G D S W E K Y V H G V R Y Q C  
 TCAAGGTGGGGAGGTGCATGCCGTTGACATTGAGGTTGACATTGAGGTTGACATTGAGGTTGACATTGAGGTTGACATTGAGGTTGACATTGAGG  
 1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680  
 13  
 Y C Y G R G I G E W H C Q P L Q Y Y P S S G P V E V F I T E T P S Q P N S H P  
 CTACTCTATGCCGTTGCGGAGTGGCATTGCCGTTGCGGAGTGGCATTGCCGTTGCGGAGTGGCATTGCCGTTGCGGAGTGGCATTGCCGTTGCG  
 1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800  
 14  
 I O W N A P Q P S H I S K Y I L R W R P K N S V G R W K E A T I P G H L N S Y T  
 CATCCAGTGGAAATGACCAACAGCAGCATCTCACATTCCAAAGTACATTCTCAGGTGGAGACCTAAATTCTGTAGGAGGAGCTTAAACTCTTACAC  
 1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 2000

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*Fig. 3C.*

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1 K G L K P G V V Y E G Q L I S I O Q Y G H Q E V T R F D F T T S T P V T  
 CATCAAAGGCTGAAAGCCTGGTGTGTTACAGGGCCAGCTCATGAGCACTGACGCCAGCTTGCACACAGCACCTGTCAC  
 1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 2040  
**15**  
 S N T V T G E T T P F S P L V A T S E S V T E I T A S S F V V S W V S A S D T V  
 CAGCAACACCGTGACAGGAGGACTCCCTTCTCTCTGGAATCTGACCGAATCAGACCCAGTAGCTGCTGCTGCTGCTGACCGT  
 2050 2060 2070 2080 2090 2100 2110 2120 2130 2140 2150 2160  
 S G F R V E Y E L S E E G D E P Q Y L D L P S T A T S V N I P D L L P G R K Y I  
 GTGGGATTGGCTGAAATAGAGCTGAGTGAAGGGAGATGAGCCAGATGAGCCAGCTGGATCTCTGACACTCTCTGACATCCCTGACAT  
 2170 2180 2190 2200 2210 2220 2230 2240 2250 2260 2270 2280  
**16**  
 V N V Y Q I S E D G E Q S L I L S T S Q T T A P D A P P D P T V D Q V D D T S I  
 TGTAATGCTCTATGATATCTGAGGATGGCAGGAGCTTGTATCTGCTGCTACTCACACACAGCAGCCCTGATGCCGACTGTGGACAGTGTGACCTCAAT  
 2290 2300 2310 2320 2330 2340 2350 2360 2370 2380 2390 2400  
**17**  
 V V R W S R P Q A P I T G Y R I V Y S P S V E G S S T E L N L P E T A N S V T L  
 TGTGTTGGAGACCCAGGCTCCATCACAGGCTACAGAATAGCTATTGCGATCATGAGGTAGCAGCACAGAACCTGCTGAACTGCTGAACTGCTGAACT  
 2410 2420 2430 2440 2450 2460 2470 2480 2490 2500 2510 2520  
 S D L Q P G V Q Y N I T I Y A V E E N Q E S T P V V I Q O E T T G T P R S D T V  
 CAGTGACTTGCACACTGGTCACTATACATCACTATCTGAGGAGAAATCAGAAGTACACCTGTTGCTTCACTGAGTGTGATGAGTGTGATAGCT  
 2530 2540 2550 2560 2570 2580 2590 2600 2610 2620 2630 2640  
**18**  
 P S P R D L Q F V E V T D V K V T I N W T P P E S A V T G Y R V D V I P V N L P  
 GCGCTTCCAGGAACTGCACTTGTGAAAGTGAAGCAGCTGAGGTCAACATCATGAGGCTAACCGCTAACGGTCACTTACCTGAGTGTGATGAGGAGGCA  
 2650 2660 2670 2680 2690 2700 2710 2720 2730 2740 2750 2760  
 G E H G Q R L P I S R N T F A E V T G L S P G V T Y Y F K V F A V S H G R E S K  
 TGCGCAAGCAGGAGGCTGCACTTGTGAGGAAACCTGAGGCTAACCGCTAACGGTCAACGGCTAACGGTCACTTACCTGAGTGTGATGAGGAGGCA  
 2770 2780 2790 2800 2810 2820 2830 2840 2850 2860 2870 2880

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Fig. 3d.

**Fig. 3e.**

E E D V A E L S I S P S O N A V V L T N L L P G T E Y V V S V S S V Y E Q H E S  
TGAGGAGATGGAGTTGTCAATTCTCCTACAGACATGAGTGGTCTACAGATTCAGAATATGTAGTAGTGGTCTACAGAGTGGTCTACAGAG  
3850 3860 3870 3880 3890 3900 3910 3920 3930 3940 3950 3960

23 T P L R G R Q K T G L D S P T G I D F S D I T A N S F T V H W I A P R A T I T G  
CACACCTCTAGAGAAAGCACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAG  
3970 3980 3990 4000 4010 4020 4030 4040 4050 4060 4070 4080

Y R I R H H P E H F S G R P R E D R V P H S R N S I T L T N L T P G T E Y V V S  
CTACAGAGATCCGCATCATCCCGAGACTTCAGTGGAGAGATCGAGAGATCGAGAGATCGAGAGATCGAGAGATCGAGAGATCGAGAGATCG  
4090 4100 4110 4120 4130 4140 4150 4160 4170 4180 4190 4200

24 I V A L N G R E E S P L L I G Q Q S T V S O V P R D L E V V A A T P T S L L I S  
CATGGTCTCTTAATGGAG  
4210 4220 4230 4240 4250 4260 4270 4280 4290 4300 4310 4320

W D A P A V T V R Y Y R I T Y G E T G G N S P V Q E F T V P G S K S T A T I S G  
CTGGGATCT  
4330 4340 4350 4360 4370 4380 4390 4400 4410 4420 4430 4440

25 L K P G V D Y T I T V Y A V T G R G D S P A S S K P I S I N Y R T E I D K P S Q  
CCTTAAACCTGGAGTTGATTATACCATCACTGTGTATCTGTCTACTGGAG  
4450 4460 4470 4480 4490 4500 4510 4520 4530 4540 4550 4560

M Q V T D V Q D N S I S V K W L P S S S P V T G Y R V T T P K N G P G P T K T  
GATGCAAGTGAAGATGGTCAAGGAAACAGCATTAGTGTCAAGTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCT  
4570 4580 4590 4600 4610 4620 4630 4640 4650 4660 4670 4680

K T A G P D Q T E M T I E G L Q P T V E Y V V S V Y A Q N P S G E S Q P L V Q T  
TAAACTGGAGTCAGATCAAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAAC  
4690 4700 4710 4720 4730 4740 4750 4760 4770 4780 4790 4800

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*Fig. 3f.*

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26  
 A V T N I D R P K G L A F T O V D V D S I K I A W E S P Q G Q V S R Y R V T Y S  
 TGGAGTAAACCAATTGATGGCCATTAGGACTGGCATTCAGTGTGATGTCATTGATGTTCAAAATTCTTGAGAAGGCCACAGGAAACAGGAACTGGTTCCAGGTACAGGTGACCTACTC  
 4810 4820 4830 4840 4850 4860 4870 4880 4890 4900 4910 4920  
  
 S P E D G I H E L F P A P D G E E D T A E L O G L R P G S E Y T V S V V A L H D  
 GACCCCTGAGATGGAAATGGAACTTACCGTAACTGAGCTTACGAGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGG  
 4930 4940 4950 4960 4970 4980 4990 5000 5010 5020 5030 5040  
  
 27  
 D M E S Q P L I G T O S T A I P A P T D L K F T Q V T P T S L S A Q W T P P N V  
 TGATATGGAGGGCCGGCCGGCTGATTGAAACCCAGTCCACACCTATTGCTGACCAACTGAGCTGAAGTTCACTCAGGGTCAACGCCAACGGCTGAGGCTGAGGCTGAGGCTGAGG  
 5050 5060 5070 5080 5090 5100 5110 5120 5130 5140 5150 5160  
  
 0 L T G Y R V R V T P K E K T G P M K E I N L A P D S S S V V V S G L M V A T K  
 TCAGCTCACTGGATATGGCTGGGGTCAACCCAGGAGAACCGAACCTGAGAAATCAACCTGGCTCTGAGCTCACTGGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAG  
 5170 5180 5190 5200 5210 5220 5230 5240 5250 5260 5270 5280  
  
 28  
 Y E V S V Y A L K D T L T S R P A Q G V V T T L E N V S P P R A R V T D A T E  
 ATATGAAAGTGGGTCTATGCTCTTAAGGAACTTTGACAGGAGACCTGAGCTCAAGGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAG  
 5290 5300 5310 5320 5330 5340 5350 5360 5370 5380 5390 5400  
  
 T T I T I S W R T K T E T I T G F Q V D A V P A N G Q T P I Q R T I K P D V R S  
 GACCAAGTACCAATTAGCTGGAGAACAGTGGAGACATCACTGGCTGGTCAAGTGGCTGAGCTCACTGGCTGAGCTCACTGGCTGAGCTCACTGGCTGAGCTCACTGGCTGAG  
 5410 5420 5430 5440 5450 5460 5470 5480 5490 5500 5510 5520  
  
 29  
 Y T I T G L Q P G T D Y K I Y L Y T L N D N A R S S P V V I D A S T A I D A P S  
 CTACACCACTCACAGGTTCACAAACCCGGCACTTACACATCTACCTGAAATGAACTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAG  
 5530 5540 5550 5560 5570 5580 5590 5600 5610 5620 5630 5640  
  
 N L R F L A T T P N S L L V S W Q P P R A R I T G Y I I K Y E K P G S P P R E V  
 CAACCTGGGTTCTGGCCACACACCCAAATTCTGGCTGAGCTGGGATTACCGGATTACCGGCTACATCAAGTATGAGGAGCTGGCTGAGCTGGCTGAGCTGGCTGAG  
 5650 5660 5670 5680 5690 5700 5710 5720 5730 5740 5750 5760

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Fig. 3g.

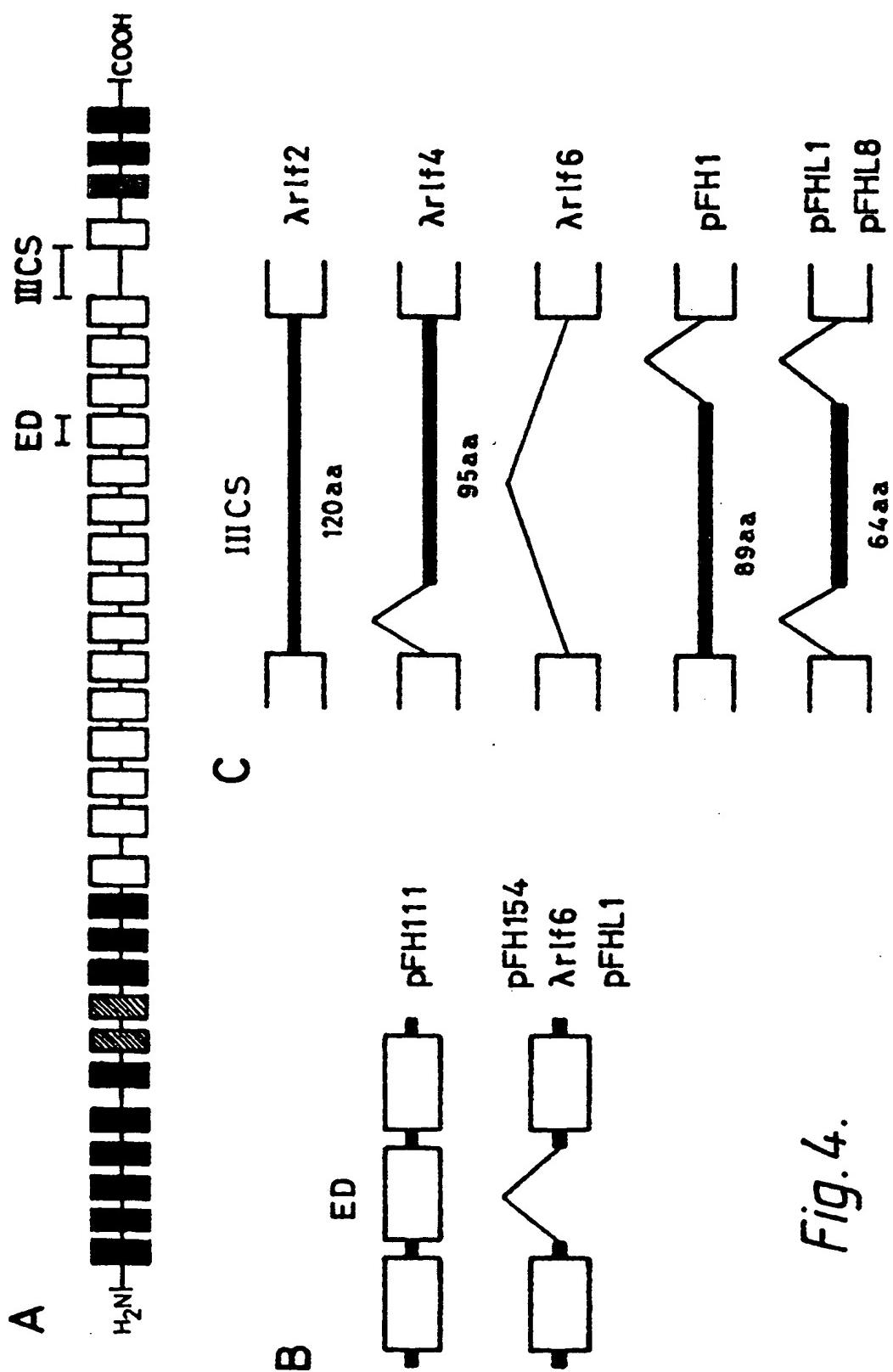
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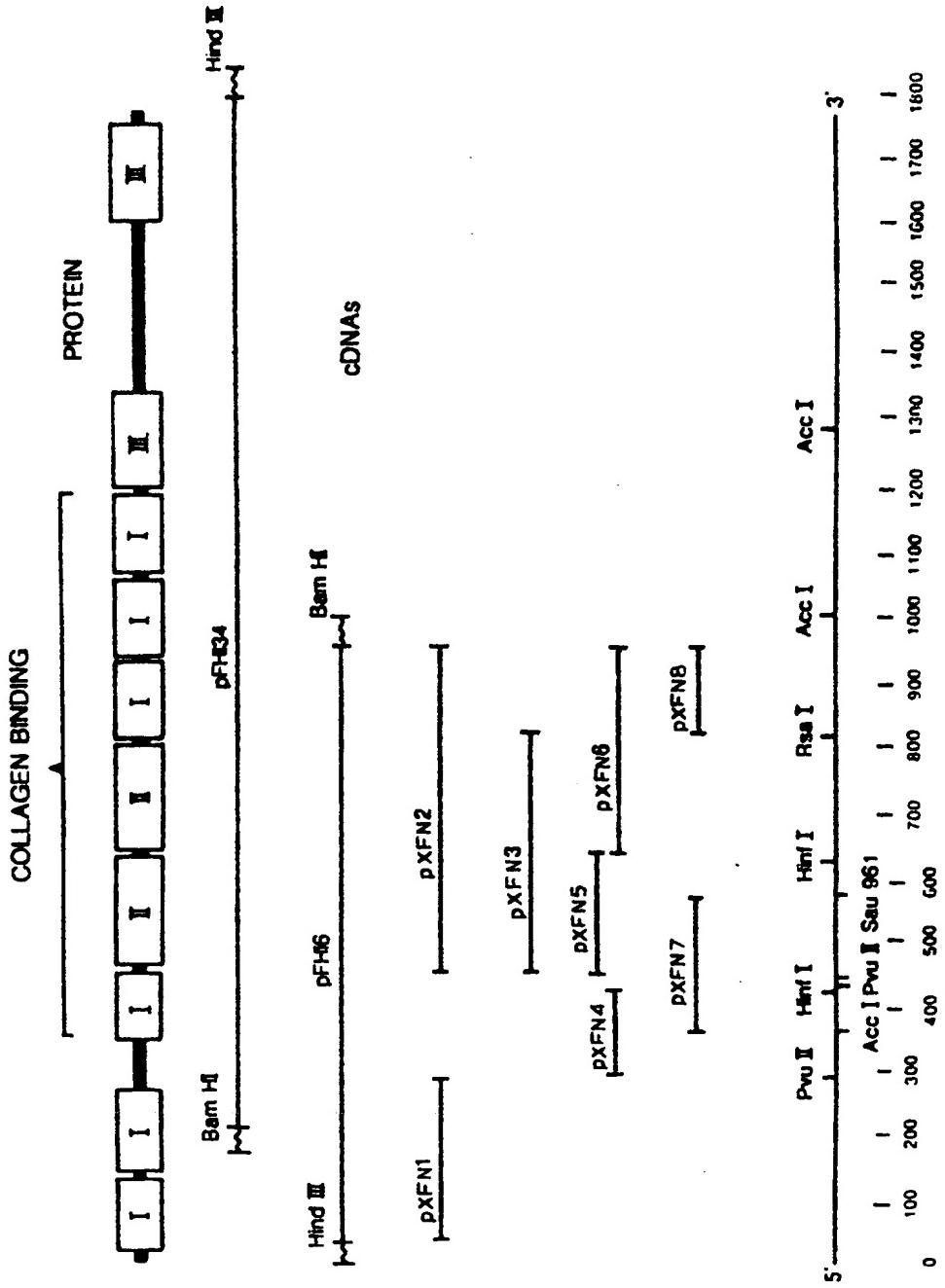
Fig. 3h.

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*Fig. 5.*



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**DOCUMENTS CONSIDERED TO BE RELEVANT**

EP 86304998.7

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int Cl.)
X	WO - A1 - 84/00 540 (LA JOLLA CANCER RESEARCH FOUNDATION) * Claims 1,10,13,22 * --	1	C 07 K 15/14 C 12 N 15/00 C 07 H 21/04 C 12 P 21/00
D,A	ANNUAL REVIEW OF BIOCHEMISTRY, vol. 52, 1983, (Palo Alto, USA) K.M. YAMADA "Cell Surface Interactions with Extracellular Materials" pages 761-799 * Pages 768-781 * --	1	C 12 N 1/20 A 61 K 37/02 //C 12 N 1/20; C 12 R 1:19)
D,A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 80, no. 11, June 1983 (Baltimore, USA) A.R. KORNBLIHTT et al. "Isolation and characterization of cDNA clones for human and bovine fibronectins" pages 3218-3222 * Totality * --	1,9	TECHNICAL FIELDS SEARCHED (Int Cl.)
D,A	CELL, vol. 35, no. 2, December 1983 (Part. 1) (Cambridge, Mass., USA) J.E. SCHWARZBAUER et al. "Three Different Fibronectin mRNAs Arise by Alternative Splicing within the Coding Region" pages 421-431 * Totality * --	1,9	C 07 K C 12 N C 07 H C 12 P
The present search report has been drawn up for all claims			
Place of search	Date of completion of the search	Examiner	
VIENNA	06-10-1986	WOLF	
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone	T : theory or principle underlying the invention		
Y : particularly relevant if combined with another document of the same category	E : earlier patent document, but published on, or after the filing date		
A : technological background	D : document cited in the application		
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P : intermediate document	& : member of the same patent family, corresponding document		



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## EUROPEAN SEARCH REPORT

**0207751**

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Application number

DOCUMENTS CONSIDERED TO BE RELEVANT			EP 86304998.7
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	<u>EP - A2 - 0 114 685 (THE GREEN CROSS CORPORATION)</u> * Pages 1-5 *	1,6,7	
A	<u>EP - A2 - 0 022 052 (AMRANI)</u> * Pages 1-5 *	1,6,7	
-----			
TECHNICAL FIELDS SEARCHED (Int. Cl.4)			
The present search report has been drawn up for all claims			
Place of search	Date of completion of the search	Examiner	
VIENNA	06-10-1986	WOLF	
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone	T : theory or principle underlying the invention		
Y : particularly relevant if combined with another document of the same category	E : earlier patent document, but published on, or after the filing date		
A : technological background	D : document cited in the application		
O : non-written disclosure	L : document cited for other reasons		
P : intermediate document	& : member of the same patent family, corresponding document		

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